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Mg^{2+} as an Extracellular Signal: Environmental Regulation of Salmonella Virulence

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Summary

Ions are not traditionally thought to act as first messengers in signal transduction cascades. However, while searching for genes regulated by the PhoP/PhoQ virulence regulatory system of Salmonella typhimurium, we recovered two loci whose expression is controlled by the concentration of Mg^{2+}. To determine whether Mg^{2+} is the signal modulating the whole PhoP/PhoQ system, we evaluated the gene expression pattern of six PhoP-activated genes. Growth in physiological concentrations of divalent cations repressed transcription of PhoP-activated genes and rendered wild-type Salmonella phenotypically $PhoP^-$. Mg^{2+} changed the conformation of the periplasmic domain of PhoQ, identifying this protein as a Mg^{2+} sensor. A mutation in the sensing domain of PhoQ altered the set point for Mg^{2+} and rendered Salmonella avirulent.

Introduction

Divalent cations are generally thought to act as second messenger molecules or as cofactors in a variety of biochemical reactions. The cytoplasmic levels of Ca^{2+}, normally at 100 nM in both eukaryotes and prokaryotes, are increased upon stimulation of a variety of receptors triggering particular signal transduction cascades (Clapham, 1995). On the other hand, Mg^{2+} is a membrane and ribosome stabilizer and is essential in reactions that require ATP (Reinhart, 1988). Indeed, the prevailing view has been that neither Ca^{2+} nor Mg^{2+} could act as extracellular signaling molecules. In this paper, however, we identify a signal transduction pathway that responds to extracellular Mg^{2+}: we show that Mg^{2+} is the signal that controls the PhoP/PhoQ two-component regulatory system of Salmonella typhimurium.

Bacterial pathogens experience different environments during the course of infection and must modulate their gene expression repertoire to survive and replicate within host tissues (Mekalanos, 1992; Dziejman and Mekalanos, 1995). This adaptation often involves two-component systems, generally consisting of a sensor/kinase and a response regulator, which use reversible protein phosphorylation to regulate the adjustment to new environments (Parkinson, 1993; Alex and Simon, 1994; Swanson et al., 1994; Parkinson and Kofoid, 1992). The sensor is often an integral membrane protein that becomes phosphorylated in response to environmental signals. The phosphate is then transferred to a conserved residue in the second component, which is often a transcription factor whose affinity for DNA is modulated by phosphorylation. The general biochemical principles governing the phosphotransfer reactions appear to be similar among the estimated 50 two-component systems that each microorganism harbors (Stock et al., 1995). However, the signals that modulate the sensors as well as the targets of transcriptional control are unique and specific for each system.

The PhoP/PhoQ two-component system controls several pathogenic properties of the facultative intracellular parasite S. typhimurium (García-Véscovi et al., 1994; Groisman and Heffron, 1995) including intramacrophage survival (Fields et al., 1986), resistance to host defense antimicrobial peptides (Fields et al., 1989; Groisman et al., 1992a, 1992b) and to acid pH (Foster and Hall, 1990), invasion of epithelial cells (Behlau and Miller, 1993), the formation of spacious vacuoles (Alpuche-Aranda et al., 1994), and the presentation of model antigens by phagocytic cells (Wick et al., 1995). *phoP* and *phoQ* encode the regulator and sensor proteins, respectively, of this regulatory system (Groisman et al., 1989; Miller et al., 1989). While expression of over 40 different proteins is modulated by PhoP (Miller and Mekalanos, 1990), the signal that controls the PhoP/PhoQ system has remained unknown.

During an exhaustive search for PhoP-regulated genes, we identified 25 loci as transcriptionally controlled by PhoP (F. C. S. et al., unpublished data). These loci included *mgtA* and *mgtCB*, encoding two high affinity Mg^{2+} transporters whose expression is increased in low Mg^{2+} media (Snavely et al., 1991). This raised the possibility that Mg^{2+} may constitute the signal governing the PhoP/PhoQ regulon. In this paper, we establish that Mg^{2+} is the environmental signal that controls PhoP/PhoQ, thereby identifying a regulatory system that responds to extracellular Mg^{2+}. We show that growth in millimolar levels of Mg^{2+} represses expression of PhoP-activated genes and attenuates the virulence properties of wild-type Salmonella. We demonstrate that PhoQ is a Mg^{2+} sensor protein whose conformation changes in the presence of periplasmic Mg^{2+}. Furthermore, we establish that altered responsiveness to Mg^{2+} results in virulence attenuation.

Results

Identification of Mg^{2+} as the Signal Controlling the PhoP/PhoQ System

To explore the possibility that Mg^{2+} is the physiological signal controlling the PhoP/PhoQ regulatory system, we investigated the expression of the PhoP-activated gene *psiD* (Groisman et al., 1989) by measuring the β-galactosidase activity originating from a *psiD–lac* transcriptional fusion. Expression of *psiD* was inversely proportional to the Mg^{2+} concentration of the growth media: maximal activation was observed in media with Mg^{2+} added to 8 μM, whereas growth in 10 mM Mg^{2+} resulted

*The first two authors contributed equally to this work.

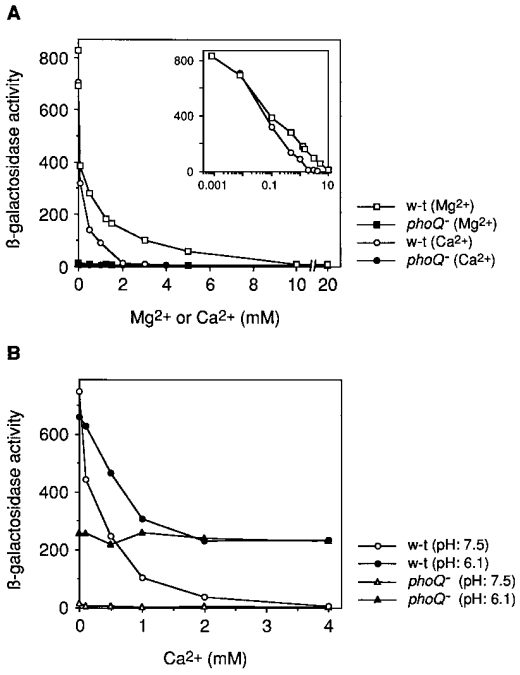

Figure 1. Divalent Cations, but Not pH, Modulate *psiD* Expression in a PhoQ-Dependent Manner

(A) β-Galactosidase activity from a *psiD–lac* transcriptional fusion expressed by wild-type and *phoQ*⁻ strains grown in N-minimal media with the indicated concentrations of $MgCl_2$ or $CaCl_2$. Inset shows the same data using logarithmic scale for the *x* axis.

(B) β-Galactosidase activity from a *psiD–lac* transcriptional fusion expressed by wild-type and *phoQ*⁻ strains grown in N-minimal media with the indicated pH and concentrations of $CaCl_2$. β-Galactosidase activity is in Miller units (Miller, 1972). The data correspond to mean values of three independent experiments done by duplicate.

in >99% repression of the highest activity (Figure 1A). The activation/repression ratio was ~100-fold, much higher than any other reported condition for the PhoP/PhoQ system. The repression effect was specific to Mg^{2+} and not to the particular counter ion, since it was observed with either $MgCl_2$ or $MgSO_4$, and was prevented by chelators of divalent cations such as EDTA. Apart from a low Mg^{2+} concentration, activation of *psiD* required functional PhoQ (Figure 1A) and PhoP (data not shown) proteins. Indeed, expression of *psiD* changed less than 2-fold when a *phoQ*⁻ mutant was grown in high versus low Mg^{2+} media (Figure 1A). These results identify Mg^{2+} as the environmental signal controlling PhoP/PhoQ and define a signal transduction pathway responding to Mg^{2+}.

Cation Specificity of PhoQ

We examined the ability of different divalent cations to modulate transcription of *psiD*. Ca^{2+} also repressed *psiD* expression in a PhoP- and PhoQ-dependent manner (Figure 1A). Half-maximal repression was attained at lower concentrations of Ca^{2+} (50 μM) than those achieved with Mg^{2+} (85 μM). Mn^{2+} could also replace Mg^{2+}, but Ni^{2+}, Cu^{2+}, or Ba^{2+} (up to 0.3 mM) had no effect. On the other hand, Co^{2+} exhibited a repressing effect only at concentrations that were toxic to the microbe. If one considers the relative abundance and toxicity of the various cations tested, then Mg^{2+} and Ca^{2+} appear to be the physiologically relevant cations controlling the PhoP/PhoQ system.

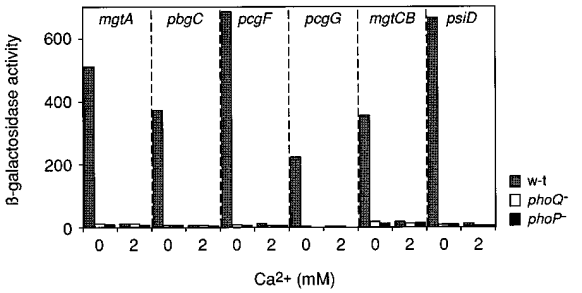

Figure 2. Ca^{2+} Represses Expression of PhoP-Activated Genes

β-Galactosidase activity expressed by strains grown in N-minimal media with or without 2 mM $CaCl_2$ was determined in strains harboring a *lac* transcriptional fusion to PhoP-activated genes (*mgtA*, *pbgC*, *pcgF*, *pcgG*, *mgtCB*, and *psiD*). The transcriptional activity was investigated in three genetic backgrouds: wild-type, *phoP*⁻, and *phoQ*⁻. Assays were performed as described in the legend to Figure 1.

Divalent Cations Repress Expression of Several PhoP-Activated Genes

If Mg^{2+} and Ca^{2+} are the physiological signals controlling the PhoP/PhoQ system, one would anticipate other PhoP-activated genes to exhibit the same regulatory behavior as *psiD*. Strains harboring *lac* transcriptional fusions to five additional PhoP-regulated genes (*mgtA*, *pbgC*, *pcgF*, *pcgG*, and *mgtCB*) exhibited the same pattern of expression under low and high Ca^{2+} (Figure 2) or Mg^{2+} (data not shown) as the *psiD–lac* strain. While the absolute values of β-galactosidase differed among these strains, the ratios of the β-galactosidase activities produced in low and high Ca^{2+} were similar to that determined for the *psiD–lac* strain. Maximal expression required functional PhoP and PhoQ proteins, and the transcriptional activities detected in their absence were similar to those displayed by the wild-type microorganism grown in high Ca^{2+} (Figure 2). On the other hand, the β-galactosidase activities exhibited by two strains harboring *lac* fusions to genes not regulated by PhoP were not affected by growth in the presence of Ca^{2+} (data not shown).

Induction Kinetics of the PhoP-Activated *psiD*

We investigated whether activation of *psiD* in vitro exhibited the same kinetics as that reported for PhoP-activated genes in vivo (García-del Portillo et al., 1992; Alpuche-Aranda et al., 1992). The Ca^{2+} chelator EGTA (6 mM) was added to bacteria growing exponentially in 2 mM Ca^{2+}, and the β-galactosidase activity originating from the *psiD–lac* fusion was determined at various times thereafter (Figure 3). The transcriptional activity of *psiD* increased exponentially during the first 40 min; by 5 hr, this activity was 180 times higher than that displayed by cells that had not received EGTA (Figure 3). The time required for maximal activation of *psiD* in vitro was similar to that displayed by PhoP-activated

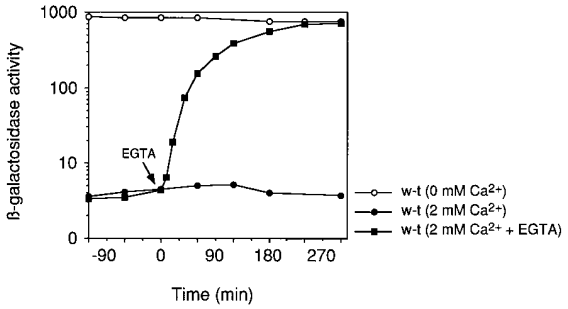

Figure 3. Induction Kinetics of the PhoP-Activated *psiD*

Bacteria were grown to logarithmic phase in N-minimal media in the presence or absence of $CaCl_2$ (2 mM). EGTA (6 mM) was added to bacteria growing in the presence of $CaCl_2$ at the point indicated by the arrow. Samples were withdrawn at the indicated times, and β-galactosidase activity was assayed as described in the Experimental Procedures.

genes in vivo, which occurs 4–6 hr after entry of the microorganism into the host cell.

Activation of PhoP-Regulated Loci by Acid pH Is Independent of PhoP and PhoQ

The induction of certain PhoP-activated genes within macrophages has been correlated with acidification of the vacuolar compartment harboring Salmonella (Alpuche-Aranda et al., 1992). While this led to the suggestion that acid pH might constitute the signal detected by PhoQ, acidification of LB broth in vitro could not stimulate PhoP-activated genes to the levels observed within macrophages (Alpuche-Aranda et al., 1992). We investigated the effect of pH on *psiD* expression and determined that its transcriptional activity also increases when the pH of the growth media is lowered from 7.5 to 6.1. However, this induction is independent of both PhoQ (see Figure 1B) and PhoP (data not shown). These results indicate that changes in pH are neither sensed by PhoQ nor transmitted to PhoP by a sensor that might respond to pH. Furthermore, that Ca^{2+} modulated *psiD* expression at both pH 6.1 and pH 7.5 substantiates the role of divalent cations in controlling the PhoP/PhoQ regulatory system.

Environmental Regulation of *phoPQ*

We have established that the *phoPQ* operon is a PhoP-activated locus and identified two *phoPQ* transcripts in cells grown in LB broth (Soncini et al., 1995). To examine whether Mg^{2+} regulates the *phoPQ* promoters, we conducted S1 mapping experiments on mRNAs prepared from bacteria grown under different conditions. A long transcript ($p_{phoP\text{-}1}$) was produced only in wild-type bacteria grown in media without added Mg^{2+} (Figure 4) and required the presence of both PhoP and PhoQ. On the other hand, the short transcript ($p_{phoP\text{-}2}$) was synthesized in wild-type, *phoP*$^-$, and *phoQ*$^-$ Salmonella, grown with either high or low Mg^{2+} concentrations (Figure 4). The production of these transcripts was also regulated by growth phase because the mRNA levels were higher in logarithmic growing cells than in those harvested from the stationary phase (Figure 4). Taken together, these results indicate that Mg^{2+} controls the positive feedback loop that regulates the *phoPQ* operon.

The PhoP/PhoQ Regulon Is Required for Growth in Mg^{2+}-Limiting Conditions

Because Mg^{2+} deprivation is the signal that activates the PhoP/PhoQ system, we reasoned that *phoP*$^-$ and *phoQ*$^-$ mutants might be impaired for growth in low Mg^{2+} media. Indeed, PhoP is necessary to activate transcription of *mgtA* and *mgtCB*, which encode the two high affinity Mg^{2+} uptake systems of S. typhimurium (Smith and Maguire, 1993; Roof and Maguire, 1994). There is a third Mg^{2+} transport system, CorA, that is constitutively expressed (i.e., its transcription does not respond to the presence of Mg^{2+} in the media). S. typhimurium strains with null alleles of *phoP* or *phoQ* could not grow on plates containing 40 μM Mg^{2+}, whereas a *corA*$^-$ mutant did (see Figure 6). As expected, strains with mutations in *corA* and *phoP* or *phoQ* did not grow on this medium either. With plates containing 200 μM Mg^{2+}, the double mutants *corA*$^-$ *phoP*$^-$ and *corA*$^-$ *phoQ*$^-$ formed small colonies, whereas strains with single mutations in any of these loci grew like the wild-type strain (Figure 5). To identify the PhoP-regulated gene(s) conferring the ability to grow in low Mg^{2+} media, we tested the behavior of the six mutants used in the gene expression studies (see Figure 2). The *pcgG* mutant could not grow in 40 μM Mg^{2+}, whereas strains with mutations in *mgtA*, *mgtCB*, *pcgF*, *pcgG*, or *psiD* grew like the wild-type strain. These results demonstrate that the PhoP/PhoQ regulon is essential for growth under Mg^{2+}-limiting conditions. Furthermore, they provide a physiological reason for Mg^{2+} being the relevant signal for PhoP/PhoQ rather than a cofactor.

Phenotypic Modulation of Peptide Resistance by Mg^{2+}

We have previously demonstrated that the PhoP/PhoQ regulon is required to resist killing by host antimicrobial peptides such as defensin NP-1 (Fields et al., 1989) and magainin 2 (Groisman et al., 1992a). When grown in LB broth, the wild-type strain is >1000-fold more resistant to these peptides than either *phoP*$^-$ or *phoQ*$^-$ mutants. These results imply that the levels of divalent cations available in LB broth must be low enough for the PhoP/PhoQ system to be active and the wild-type strain to display the peptide resistance phenotype. Therefore, we predicted that growth in high concentrations of Mg^{2+} would render wild-type Salmonella phenotypically PhoP$^-$ (i.e., sensitive to antimicrobial peptides). Indeed, when the wild-type strain was grown in LB broth supplemented with Mg^{2+} (25 mM), it was >1000-fold more susceptible to magainin 2 than bacteria grown without added Mg^{2+} (Figure 6), and its susceptibility approached that exhibited by the *phoP*$^-$ mutant. As expected, the hypersusceptibility of the *phoP*$^-$ strain toward magainin 2 was not altered by the presence of Mg^{2+} in the growth media. These results indicate that Mg^{2+} modulates complex phenotypic properties, such as resistance to antimicrobial peptides, in a PhoP-dependent fashion.

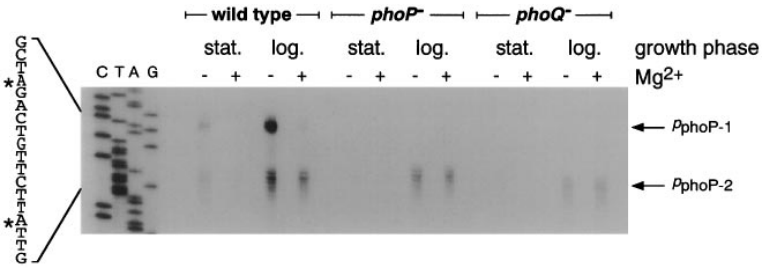

Figure 4. Environmental Regulation of *phoPQ* Operon

S1 mapping of the *phoPQ* transcripts produced in wild-type (14028s), *phoP*$^-$ (MS7953s), and *phoQ*$^-$ (MS5996s) strains grown in LB broth with (plus) or without (minus) $MgCl_2$ (25 mM) and harvested during the logarithmic (log) or stationary (stat) phases. The S1 protection was performed as described in the Experimental Procedures. p_{phoP-1} and p_{phoP-2} indicate the two protected transcripts. Lanes C, T, A, and G correspond to dideoxy-chain termination sequence reactions corresponding to this region. The asterisks indicate transcription start sites.

PhoQ Responds to Periplasmic Mg^{2+}

The PhoQ protein features two transmembrane regions, a long cytoplasmic tail, and a large periplasmic domain rich in acidic residues that could be involved in binding Mg^{2+} (Figure 7A). To examine whether PhoQ responds to periplasmic (rather than cytoplasmic) Mg^{2+}, we investigated the behavior of a chimera consisting of the N-terminal (periplasmic) domain of EnvZ and the C-terminal (cytoplasmic) domain of PhoQ (EnvZ is a PhoQ homolog that mediates the response to osmolarity). This chimera (ZhoQ) rescued a *phoQ*$^-$ mutant for transcriptional activation of *psiD* in both high and low Mg^{2+} media, indicating that the periplasmic domain of PhoQ is necessary to mediate the repressing effect of Mg^{2+}. That PhoQ responds to periplasmic Mg^{2+} is further substantiated by the constancy of the intracellular Mg^{2+} concentration:

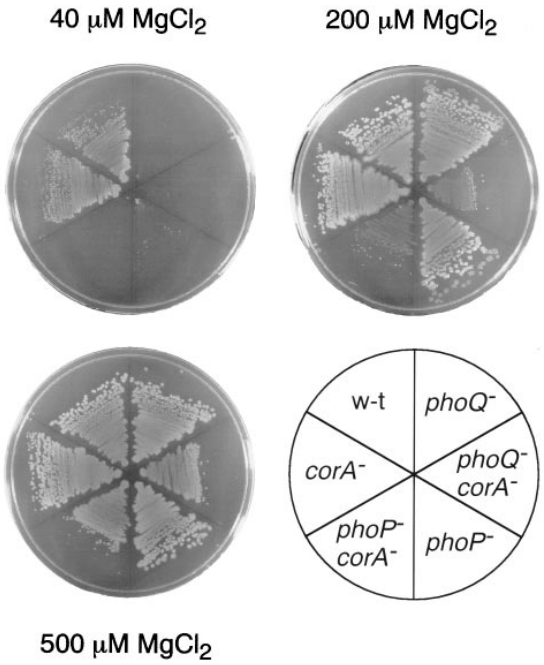

Figure 5. *phoP*$^-$ and *phoQ*$^-$ Mutants Are Impaired for Growth in Low Mg^{2+} Media

Bacterial strains (wild type [14028s], *phoQ*$^-$ [MS5996s], *phoP*$^-$ [MS7953s], *corA*$^-$ [EG9586], *phoQ*$^-$ *corA*$^-$ [EG9612], and *phoP*$^-$ *corA*$^-$ [EG9611]) were streaked onto N-minimal media agarose plates, with the addition of $MgCl_2$ to 40 μM, 200 μM, or 500 μM final concentrations, and incubated at 37°C for 36 hr before photography.

it changed less than 2-fold when the extracellular concentration of Mg^{2+} varied over 1000-fold (Silver and Clark, 1971; our unpublished data). Cumulatively, these results indicate that PhoQ is a sensor that responds to periplasmic Mg^{2+} levels.

Mg^{2+} Modifies the Conformation of the Periplasmic Domain of PhoQ

To examine whether Mg^{2+} may directly alter the conformation of PhoQ, we investigated the ability of Mg^{2+} to modify the trypsin susceptibility of this protein. Spheroplasts were prepared from Escherichia coli cells that expressed the S. typhimurium PhoQ and were incubated with trypsin in the presence or absence of Mg^{2+}. The protein samples were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and the PhoQ-derived fragments were identified by Western blot analysis using antibodies raised against the cytoplasmic domain of PhoQ (Figure 7B). Mg^{2+} altered the trypsinization pattern of PhoQ: spheroplasts that were incubated in the absence of Mg^{2+} exhibited a distinct profile that was characterized by the rapid disappearance of full-size PhoQ and the appearance of smaller PhoQ-derived

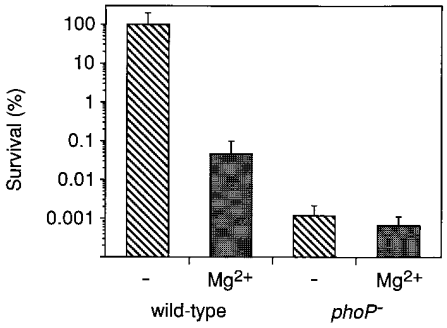

Figure 6. Growth in High Mg^{2+} Concentrations Renders Wild-Type S. typhimurium Susceptible to Magainin 2

Wild-type (14028s) and *phoP*$^-$ (MS7953s) cells were grown to logarithmic phase in LB broth in the presence (Mg^{2+}) or absence (minus) of $MgCl_2$ (25 mM). Magainin 2 was added to washed bacteria at a final concentration of 100 μg/ml, and incubated for 1 hr at 37°C. Samples were diluted and plated on LB agar plates to assess bacterial viability. Survival values are relative to the original inoculum. They were normalized by considering the wild-type strain grown without added Mg^{2+} as 100%. Note logarithmic scale of the *y* axis. Data correspond to mean values of three independent experiments done by duplicate.

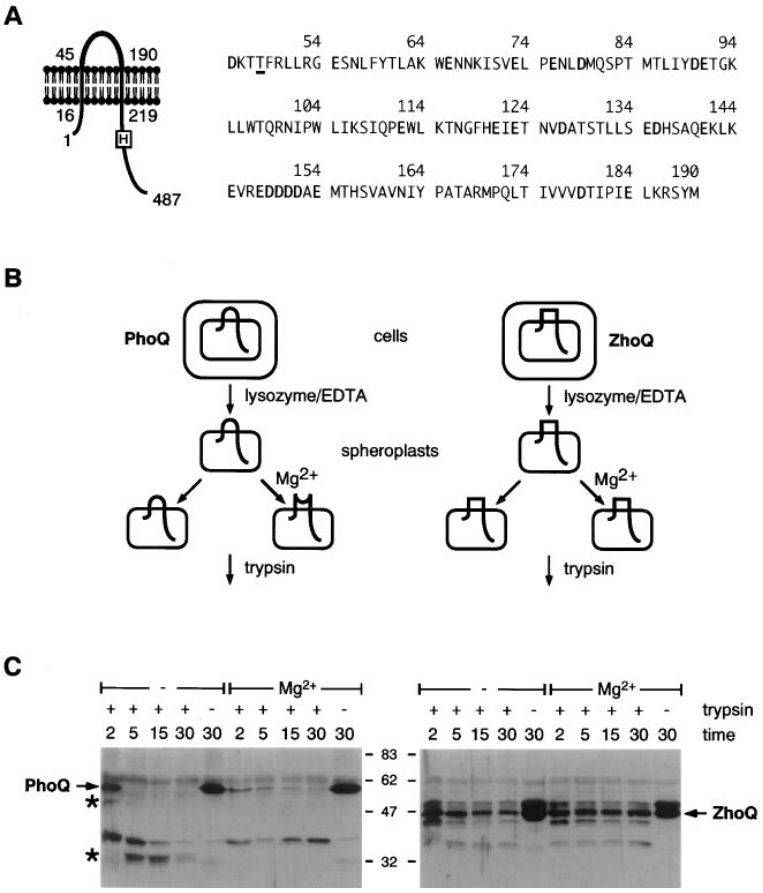

Figure 7. Mg^{2+} Modifies the Conformation of the Periplasmic Domain of PhoQ

(A) Predicted topology of PhoQ and amino acid sequence of its periplasmic domain. Characters in bold indicate acidic residues that could be involved in the interaction with Mg^{2+}. Underlined T is substituted by I in the *pho-24* mutant.

(B) Trypsin sensitivity assay. Experimental approach used to investigate the ability of Mg^{2+} to modify the trypsin susceptibility of PhoQ and ZhoQ. E. coli *phoP*$^-$ cells expressing either *phoQ* or *zhoQ* were grown overnight in LB broth, and spheroplasts were prepared by the lysozyme/EDTA method as described in the Experimental Procedures. Spheroplasts were equilibrated in the presence or absence of $MgCl_2$ (15 mM) and then treated with 0.25 mg/ml trypsin. Proteins were precipitated with TCA and analyzed by SDS–PAGE and immunoblotting.

(C) Western blot analysis of PhoQ-containing fragments following trypsin treatment. Nitrocellulose membranes containing protein samples corresponding to trypsin-treated spheroplasts were developed using anti-PhoQ (C-terminal cytoplasmic domain) polyclonal antibodies. The inmunoblot shows the products resulting from proteolysis of PhoQ (left) and ZhoQ (right). Aliquots of trypsin-treated (plus) samples in the absence or presence of $MgCl_2$ (15 mM) were withdrawn at 2, 5, 15, and 30 min. Minus signs denote controls to assess protein stability after 30 min of incubation in the absence of trypsin. The values between immunoblots indicate the position of molecular mass marker proteins. The arrows indicate full-size PhoQ and ZhoQ proteins. Asterisks indicate proteolytic products of PhoQ that specifically appear in samples incubated in the absence of Mg^{2+}.

fragments (Figure 7C). Differences in trypsin susceptibility could be seen even in incubation periods as short as 2 min (Figure 7C). The Mg^{2+}-dependent protection of PhoQ was observed at the same concentration required to repress expression of PhoP-activated genes (see Figure 1A). This concentration of Mg^{2+} did not modify the activity of trypsin toward an exogenous substrate (BAEE).

Several controls were performed to assure the integrity of the spheroplasts and the specificity of the interaction between Mg^{2+} and PhoQ. First, during the time of the assay, Mg^{2+} did not alter the integrity of the spheroplasts as determined by optical density and by the trypsin susceptibility of the cytoplasmic PhoP protein that was coexpressed with PhoQ (data not shown). Second, Mg^{2+} did not affect the stability of PhoQ in spheroplasts that were not treated with trypsin (Figure 7C). Third, the trypsin susceptibility of the ZhoQ chimera, which is blind to Mg^{2+} (see above), was not affected by the presence of Mg^{2+} (Figure 7C). In sum, these results provide direct evidence that physiological concentrations of extracytoplasmic Mg^{2+} can specifically change the conformation of PhoQ. Furthermore, this conformational change does not require additional soluble components from the periplasmic space, since it was detected in spheroplasts.

An Avirulent Mutant Is Less Responsive to Mg^{2+} Owing to a Mutation in the Periplasmic Domain of PhoQ

An S. typhimurium strain harboring the *pho-24* allele overexpresses several PhoP-activated genes and is attenuated for mouse virulence (Miller and Mekalanos, 1990). This mutation has been mapped to the *phoP* locus (Kier et al., 1979), yet no physical alterations could be detected in the *phoPQ* operon by the polymerase chain reaction (PCR) (data not shown). To establish the molecular basis for the mutant phenotype, we determined the DNA sequence of *phoPQ* and identified a single nucleotide change resulting in a single amino acid substitution: Thr-48→Ile, corresponding to the periplasmic domain of PhoQ (Figure 7A). We explored the possibility of virulence attenuation resulting from an altered response of the mutant PhoQ to Mg^{2+}. Expression of the PhoP-activated gene *psiD* was repressed by increasing concentrations of divalent cations in both *pho-24* and *phoQ*$^+$ strains (Figure 8). However, the *pho-24* mutant was less sensitive to Mg^{2+} and Ca^{2+}, and higher concentrations of these cations were necessary to achieve repression (Figure 8). These results establish that altered Mg^{2+} sensing leads to avirulence. Moreover, they provide further evidence for a direct involvement of the periplasmic domain of PhoQ in sensing Mg^{2+}.

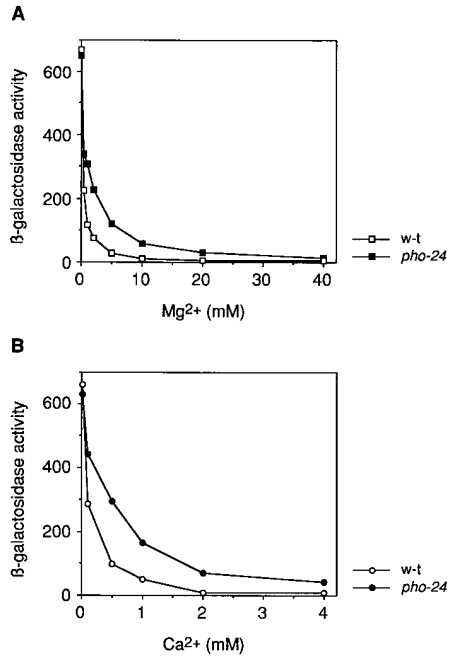

Figure 8. An Attenuated Strain Harboring a Mutation in the Periplasmic Region of PhoQ Is Less Responsive to Divalent Cations

(A) β-Galactosidase activity from a *psiD–lac* transcriptional fusion expressed by wild-type and *pho-24* strains grown in N-minimal media with the indicated concentrations of $MgCl_2$.

(B) β-Galactosidase activity from a *psiD–lac* transcriptional fusion expressed by wild-type and *pho-24* strains grown in N-minimal media with the indicated concentrations of $CaCl_2$. β-Galactosidase activity is in Miller units (Miller, 1972). The data correspond to mean values of three independent experiments done by duplicate.

Discussion

Mg^{2+} is the second most abundant cation within cells and the fourth most abundant total cation. While it has been implicated in numerous physiological processes (Reinhart, 1988) and is an essential cofactor in all phosphoryl transfer reactions that involve ATP, our experiments provide a singular example of Mg^{2+} acting as a specific signaling molecule. Extracellular Mg^{2+} controls the PhoP/PhoQ regulatory system by acting as a ligand of the sensor protein PhoQ. Indeed, Mg^{2+} changed the conformation of the periplasmic domain of wild-type PhoQ (Figure 7C) modulating the transcriptional activity of PhoP in vivo (Figure 1A), and a strain with a null allele of *phoQ* could not respond to changes in extracellular Mg^{2+}. Sensing requires the periplasmic domain of PhoQ, since a chimera harboring the periplasmic region of EnvZ fused to the cytoplasmic domain of PhoQ (ZhoQ) lost the capacity to respond to Mg^{2+}. Moreover, a mutant harboring a single amino acid substitution in the periplasmic region of PhoQ (Figure 7A) was less responsive to Mg^{2+} (Figure 8). Furthermore, the periplasmic domain of PhoQ (Figure 7A) has several acidic residues that could form a carboxylate cluster akin to that responsible for Mg^{2+} binding in the bacterial chemotaxis protein CheY (Stock et al., 1989; Volz and Matsumura, 1991). That the intracellular Mg^{2+} concentration changed less than 2-fold when the external Mg^{2+} levels varied >1000-fold (Silver and Clark, 1971; our unpublished data) argues against Mg^{2+} acting as a cytoplasmic signal.

Our results demonstrate that Mg^{2+} is not a cofactor, but rather, the physiological signal controlling the PhoP/PhoQ regulatory system. This conclusion is based on the following. First, the high induction of transcriptional activity achieved for several PhoP-activated genes by growth in low concentrations of divalent cations (Figures 1A and 2). This induction is dependent on both the sensor PhoQ and the regulator PhoP. Second, the identification of *mgtA* and *mgtCB*, encoding two of the three Mg^{2+} uptake systems of S. typhimurium (Smith and Maguire, 1993; Roof and Maguire, 1994), as transcriptionally regulated by PhoP. Third, the inability of *phoP*$^-$ and *phoQ*$^-$ mutants to grow in low Mg^{2+} media (Figure 5), and the identification of the PhoP-activated gene *pbgC* as necessary for this ability. Fourth, the phenotypic conversion of wild-type Salmonella to PhoP$^-$ resulting from growth in repressing concentrations of Mg^{2+} (Figure 6). Cumulatively, these results establish PhoP/PhoQ as a regulon governing the response to Mg^{2+} deprivation.

Both Mg^{2+} and Ca^{2+} repressed transcription of PhoP-activated genes. The regulatory role of Mg^{2+} can be rationalized in terms of PhoP/PhoQ controlling two high affinity Mg^{2+} uptake systems (Snavely et al., 1991) and a gene that is essential for growth in Mg^{2+}-limiting conditions. On the other hand, the Ca^{2+} effect may just be due to the intrinsic nature of Mg^{2+}-binding sites. It has recently been suggested that differences in the binding sites for Mg^{2+} and Ca^{2+} can be ascribed to their different coordination environments (Needham et al., 1993). Ca^{2+}-binding sites disfavor ions, such as Mg^{2+}, that possess coordination numbers different from that of Ca^{2+}. On the other hand, the adjustable nature of the Mg^{2+}-binding sites can accommodate ions of different sizes including Ca^{2+}. Alternatively, the molecular characterization of other members of the PhoP regulon may help explain the regulatory role of Ca^{2+}. This cation has been implicated in the control of several bacterial processes (Norris et al., 1991) including virulence in Yersinia (Straley et al., 1993).

While our work demonstrated that Mg^{2+} controls the PhoP/PhoQ regulon in vitro, we expect the same signals to modulate expression of this regulon during the course of infection. Indeed, the induction kinetics of *psiD* in vitro (Figure 3) was similar to that exhibited by other PhoP-activated genes in vivo. Maximal induction of *mgtCB* within epithelial cells (García-del Portillo et al., 1992) and *pagC* within macrophages (Alpuche-Aranda et al., 1992) also occurs 4–6 hr after internalization of the microorganism. While the time of *pagC* expression has been correlated with phagosome acidification, several experiments argue against acid pH being the signal controlling PhoP/PhoQ. First, the stimulation of the PhoP-activated gene *psiD* by acid pH was independent of both PhoP and PhoQ (Figure 1B). Second, Ca^{2+} modulated *psiD* expression in a PhoQ-dependent manner at both neutral and acid pH (Figure 1B). Third, recent data indicates that 95% of the phagosomes that harbor Salmonella acidify by 30 min (Rathman et al., 1995), not 5 hr (Alpuche-Aranda et al., 1992).

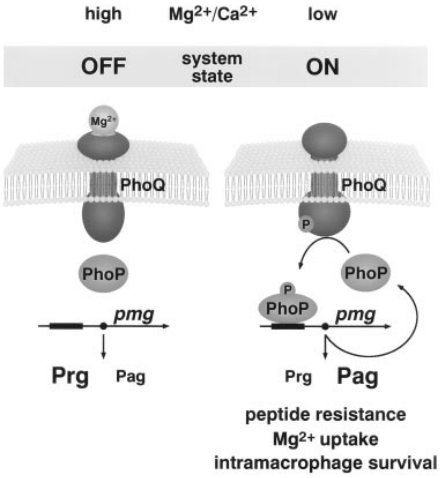

Figure 9. Model for the Regulation of the Salmonella PhoP/PhoQ Two-Component System by Divalent Cations

In the presence of millimolar (high) concentrations of Mg^{2+}, PhoQ adopts a conformation that favors its phosphatase activity or inhibits its kinase activity (or both). This leads to a decrease in the amount of phospho-PhoP reducing expression of PhoP-activated (Pag) genes. In this state (OFF), the PhoP-repressed (Prg) genes are transcriptionally active. In a Mg^{2+}-deprived environment (low), the conformation of PhoQ favors its kinase activity or inhibits its phosphatase activity (or both), increasing the concentration of phospho-PhoP. This activates Pag genes including *phoPQ* creating a positive feedback loop that amplifies the response. In this state (ON), the Prg genes are transcriptionally inactive. The ON state confers the bacteria virulence-related phenotypes such as resistance against antimicrobial peptides and the ability to survive inside macrophages.

Expression of the PhoP/PhoQ regulon is predicted to vary during the course of infection. When S. typhimurium is present in extracellular fluids, the system would be off (Figure 9) because the concentrations of Mg^{2+} and Ca^{2+} in serum are in the 0.7–1.0 mM (Reinhart, 1988) and 1–1.3 mM (Brown, 1994) range, respectively. The presence of these divalent cations would promote PhoQ conformations that favor its phosphatase activity, inhibit its kinase activity, or both. This would lead to a decrease in the amount of phosphorylated PhoP, resulting in the reduction of PhoP-activated determinants and the appearance of PhoP-repressed products. Once inside a cell, Salmonella resides within membrane-bound vacuoles (i.e., phagosomes) and is not exposed to the 0.5 mM Mg^{2+} in the cytosol. This suggests that during phagosome maturation the concentration of Mg^{2+} decreases to levels that are low enough to turn the PhoP/PhoQ system on. Indeed, the phagosomal concentrations of Mg^{2+} and Ca^{2+} have been estimated at <50 μM for epithelial cells harboring Salmonella (García-del Portillo et al., 1992) and <100 μM for macrophages containing Yersinia pestis (Pollack et al., 1986), respectively. Low levels of divalent cations would result in transcriptional activation of PhoP-activated genes including *phoPQ* (Figure 4), creating a positive feedback loop that would produce even higher levels of PhoP-activated products (Figure 9). Our work also demonstrates that the ability to sense Mg^{2+} is paramount to the pathogenicity of Salmonella: a *phoQ* mutant that was less responsive to Mg^{2+} (Figure 8) was as attenuated for virulence as a strain with a null allele of *phoQ* (Miller and Mekalanos, 1990).

The identification of a ubiquitous cation as the signal molecule controlling the PhoP/PhoQ system accounts for the wide phylogenetic distribution of *phoPQ* among gram-negative bacteria (Groisman et al., 1989; our unpublished data), which includes species not known to reside within host cells. Furthermore, it rules out earlier proposals that the cues controlling PhoP/PhoQ are specific to macrophages (Alpuche-Aranda et al., 1992). It would appear that the PhoP/PhoQ system is ancestral to enteric bacteria in which it has been used for a basic physiological process: the response to Mg^{2+} deprivation. For example, the Mg^{2+} transporters MgtA and MgtB are also present in nonpathogenic E. coli. Other PhoP-regulated genes, such as *pagC*, are specific to Salmonella and endow the microbe with the capacity to prosper within host tissues. Their recruitment under PhoP control would insure the proper temporal and spacial expression of these virulence determinants.

Finally, ions were traditionally thought to act as second messenger molecules (Clapham, 1995) and not to initiate signal transduction cascades. This was especially true for Mg^{2+} and Ca^{2+} owing to the high concentration of these divalent cations in extracellular fluids. However, we have now demonstrated that extracellular Mg^{2+} can signal PhoQ to modulate transcription of a large set of genes that mediate the adaptation to a low Mg^{2+} environment. That extracellular Ca^{2+} can also play a regulatory role has recently been shown with the characterization of the mammalian parathyroid Ca^{2+}-sensing receptor (Brown et al., 1993). This receptor can sense a decrease in the concentration of extracellular Ca^{2+} to initiate a signal transduction cascade that results in increased secretion of parathyroid hormone by parathyroid cells (Hebert and Brown, 1995). Both PhoQ and the parathyroid Ca^{2+}-sensing receptor are precise sensing devices because mutations that raise the set point for their respective extracellular cations attenuate virulence in pathogenic bacteria (this work) and lead to inherited hypocalcemia in humans (Pollak et al., 1994). In sum, Mg^{2+}, Ca^{2+}, and potentially other ions need now be considered as extracellular signaling molecules in addition to their roles as cofactors or second messengers.

Experimental Procedures

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in Luria broth (LB; Miller, 1972), or in N-minimal media containing 0.1% casaminoacids, 38 mM glycerol, and 8 μM $MgCl_2$ (Snavely et al., 1991). Ampicillin and IPTG were used at 50 μg/ml and 0.7 mM, respectively.

Bacterial Genetic and Molecular Biology Techniques

Phage P22–mediated transductions were performed as described (Davis et al., 1980). Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus following the recommendations of the manufacturer. Recombinant DNA techniques were performed according to standard protocols (Sambrook et al., 1989). The chimeric *zhoQ* gene was cloned under control of the p_{lac}-derivative promoter, between the BamHI and HindIII sites of pUHE21–2*lacI*^{q}. ZhoQ consists of the 184 N-terminal amino acids from S. typhimurium EnvZ fused to 268 C-terminal amino acids of PhoQ. To construct *zhoQ*, we ligated two DNA segments to plasmid

Table 1. Bacterial Strains and Plasmids

Bacterial Strains	Description[a]	Reference
S. typhimurium		
14028s	Wild type	Fields et al. (1986)
MS7953s	*phoP7953::Tn10*	Fields et al. (1989)
MS5996s	*phoQ5996::Tn10*	Fields et al. (1989)
EG9065	*psiD::Mu dJ*	This work
EG9042	*phoP7953::Tn10 psiD::Mu dJ*	This work
EG9461	*phoQ5996::Tn10 psiD::Mu dJ*	This work
EG9564	*pho-24; psiD::Mu dJ*	This work
EG9521	*mgtA9226::Mu dJ*	This work
EG9522	*phoQ5996::Tn10 mgtA9226::Mu dJ*	This work
EG9523	*phoP7953::Tn10 mgtA9226::Mu dJ*	This work
EG9527	*mgtCB9232::Mu dJ*	This work
EG9528	*phoQ5996::Tn10 mgtCB9232::Mu dJ*	This work
EG9529	*phoP7953::Tn10 mgtCB9232::Mu dJ*	This work
EG9524	*pbgC9228::Mu dJ*	This work
EG9525	*phoQ5996::Tn10 pbgC9228::Mu dJ*	This work
EG9526	*phoP7953::Tn10 pbgC9228::Mu dJ*	This work
EG9532	*pcgG9283::Mu dJ*	This work
EG9533	*phoQ5996::Tn10 pcgG9283::Mu dJ*	This work
EG9534	*phoP7953::Tn10 pcgG9283::Mu dJ*	This work
EG9530	*pcgF9281::Mu dJ*	This work
EG9531	*phoQ5996::Tn10 pcgF9281::Mu dJ*	This work
EG9532	*phoP7953::Tn10 pcgF9281::Mu dJ*	This work
EG9586	*corA27*	This work
EG9611	*corA27 phoP7953::Tn10*	This work
EG9612	*corA27 phoQ5996::Tn10*	This work
E. coli		
FS1002	*F⁻ araD139 Δ(lac)U169 rpsL150 relA1 thi flbB5301 deoC1 ptsF25 rbsR phoP::kan*	Groisman et al. (1992a)
Plasmids		
pUHE21–2*lacI*^{q}	*rep*$_{pMB1}$ *Ap*^{r} *lacI*^{q}	Soncini et al. (1995)
pEG9071	*rep*$_{pMB1}$ *Ap*^{r} *lacI*^{q} *phoPQ*$^+$	Soncini et al. (1995)
pEG9090	*rep*$_{pMB1}$ *Ap*^{r} *lacI*^{q} *zhoQ*$^+$	This work
pEG5381	*rep*$_{pMB1}$ *Ap*^{r} *phoPQ*$^+$	Groisman et al. (1989)

[a] Gene designations are as summarized by Sanderson et al. (1995).

pUHE21–2*lacI*^{q}: a 654 bp *envZ*-derived fragment generated by the PCR using the primers *310*, 5′-CGGGATCCGATGAGGCGAATG CGC-3′, and *311*, 5′-AAGGCCTATTCTGTATACGAATAAA-3′. The second fragment consisted of a 804 bp fragment generated by the PCR using the primers *279*, 5′-GGAATTCCATATGAGGCCTATCGA GGCG-3′, and *265*, 5′-TATAAAGCTTATTCCTCTTTCTGTGT-3′. To determine the nucleotide sequence of the *phoPQ* operon in mutant *pho-24*, we used the AmpliCycle sequencing kit (Perkin Elmer) on overlapping PCR-generated products.

The S1 protection assay was performed as described (Aiba, 1983) with RNA from logarithmic (OD_{600}, 0.9–1) or stationary phase cells grown in LB in the presence or absence of 25 mM $MgCl_2$. We used a PCR product generated with primers *312* and *369* (Soncini et al., 1995) that was labeled at the 5′ end by phosphorylation with [γ-^{32}P]ATP using T4 polynucleotide kinase (GIBCO/BRL) as DNA probe. This fragment encompasses both transcription start sites of the *phoPQ* operon. In brief, RNA (50 μg) and the probe DNA were mixed together in 50 μl of hybridization buffer (80% formamide, 20 mM HEPES [pH 6.5], 0.4 M NaCl). The mixture was incubated at 75°C for 10 min and left to cool down in an incubator at 37°C overnight. After adding 220 μl of H_2O, 30 μl of 10× S1 nuclease buffer (0.3 M sodium acetate [pH 4.5], 0.5 M NaCl, 10 mM $ZnSO_4$, 50% glycerol), the mixture was treated with 10 U of S1 nuclease (Promega) for 30 min. The reaction was stopped by adding 300 μl of phenol–chloroform, and the aqueous phase was precipitated with ethanol. The precipitate was dissolved in sequencing loading buffer and electrophoresed in 6% acrylamide sequencing gel.

Spheroplasts Preparation, Trypsinization, and Western Blot Analysis

Overnight cultures of E. coli FS1002 carrying either plasmid pEG9071 or pEG9090 were used to inoculate fresh LB media containing 50 μg/ml ampicillin and 0.7 mM IPTG. Following growth to log phase at 37°C with constant shaking, cells were washed twice with 10 mM Tris–HCl (pH 8.0) and resuspended in 20% sucrose, 30 mM Tris–HCl (pH 8.0). Spheroplasts were prepared by adding lysozyme (10 μg/ml) and EDTA (1 mM) to the resuspended cells. The efficiency of spheroplasts formation (more than 90%) was monitored by light microscopy and by recording OD_{600} after lysing an aliquot in a hiposmotic solution. After allowing to equilibrate for 10 min in the presence or absence of $MgCl_2$ (15 mM), the spheroplast preparations were treated with trypsin (0.25 mg/ml) for different periods of time. All procedures were performed at 4°C. Proteolysis was stopped on dry ice by addition of freshly prepared PMSF (1 mM). Proteins were precipitated with 10% TCA, resuspended in denaturing loading buffer, and boiled 3 min prior to loading. SDS–PAGE was performed by the method of Laemmli (1970), using 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (BioBlot-NC, Costar) and probed with affinity-purified rabbit polyclonal antibodies raised against the C-terminal cytoplasmic domain of PhoQ. Proteins detected by the primary antibody were visualized by the use of an enhanced chemiluminescence assay (ECL) using reagents from Amersham and by exposure to film. The effect of Mg^{2+} (15 mM) on trypsin activity was evaluated using the chromogenic exogenous substrate N-α-benzoyl-L-arginine ethyl ester (BAEE [Sigma]) as previously described (Bergmeyer et al., 1974).

Peptide Resistance and β-Galactosidase Assay

Susceptibility to magainin 2 (Bachem) was evaluated as previously described (Groisman et al., 1992a). In brief, bacteria were grown at 37°C in LB broth with or without $MgCl_2$ (25 mM) to early logarithmic phase. Before the addition of magainin 2 (to 100 μg/ml), $MgCl_2$ was added to all cultures to 25 mM, and the cells were washed in LB broth and diluted to 5×10^4 to 1×10^5 CFU/ml in LB broth. After 1 hr incubation, bacteria were diluted and plated onto LB agar plates,

and the number of colony forming units was determined after overnight incubation. Data are presented as percent survival relative to the original inoculum. β-Galactosidase activity was determined as described by Miller (1972).

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